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(54) Title: IDENTIFICATION AND USE OF ANTIVIRAL COMPOUNDS (57) Abstract The present invention relates to the identification of host cell proteins that interact with viral proteins required for virus replication, and high throughput assays to identify compounds that interfere with the specific interaction between the viral and host cell protein. Interfering compounds that inhibit viral replication can be used therapeutically to treat viral infection. The invention is based, in part, on the Applicants' discovery of novel interactions between proteins of the influenza virus and human host cell proteins. One of these host cell proteins, referred to herein as NPI-1, interacts with influenza virus protein NP, and may be an accessory protein required for replication of influenza virus. Another of these host cell proteins, referred to herein as NSII-1, interacts with influenza virus protein NS ₁ . Compounds that interfere with the binding of the host cell and viral proteins, and inhibit viral replication can be useful for treating viral infection <i>in vivo</i> .		

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IDENTIFICATION AND USE OF ANTIVIRAL COMPOUNDS

5 This application is a continuation-in-part of co-
pending application Serial No. 08/246,583, filed May
20, 1994, which is hereby incorporated by reference in
its entirety.

1. INTRODUCTION

10 The present invention relates to the
identification of new cellular targets for viral
intervention, the identification of antiviral
compounds that act on the new targets, and the
therapeutic use of such antiviral compounds.

15 2. BACKGROUND OF THE INVENTION

Influenza A virus is a negative strand RNA virus
belonging to the orthomyxovirus family. The genome of
the virus consists of 8 segments and encodes 10
polypeptides. Experimental evidence generated in the
20 laboratory of Scholtissek indicates that the
nucleoprotein (NP) is a major determinant of species
specificity of influenza viruses (Scholtissek, et al.,
1985, Virology 147: 287-294). Phylogenetic analysis
divides NP genes into two families: one containing NPs
25 predominantly of avian origin, and one containing
those of human origin (Bean, 1984, Virology 133:438-
442; Buckler-White & Murphy, 1986, Virology 155: 345-
355; Gammel, et al., 1989, Virology 170:71-80;
Scholtissek, et al., 1985, *supra*). The human virus
30 A/HK/1/68 and viruses having genetically related NPs
cannot rescue mutants of the avian virus
A/FPV/Rostock/1/34 with ts defects in the NP following
double infection of chicken embryo fibroblasts (CEF)
at 40°C (Scholtissek, et al., 1985, *supra*;
35 Scholtissek, et al., 1978, Virology 91: 79-85).
However, the human viruses which failed to rescue the

ts mutants on CEF cells were able to do so on Madin-Darby canine kidney(MDCK) cells (Scholtissek, et al., 1978, *supra*). Additionally, A/HK/1/68 virus and A/FPV/Rostock/1/34 virus reassortants containing the A/HK/1/68 virus-derived NP replicate in MDBK cells but
5 not in CEFs (Scholtissek, et al., 1978, *supra*). The host-specific rescue of FPV ts mutants and the host restriction of A/HK/1/68 virus reassortants suggest that a factor(s) of host origin, which differs between mammalian and avian cells, is responsible for this
10 phenomenon, and that this factor may interact with the influenza A virus NP. However, heretofore, no host protein has been identified.

Replication and transcription of influenza virus RNA requires four virus encoded proteins: the NP and
15 the three components of the viral RNA-dependent RNA polymerase, PB1, PB2 and PA (Huang, et al., 1990, J. Virol. 64: 5669-5673). The NP is the major structural component of the virion which interacts with genomic RNA, and is required for antitermination
20 during RNA synthesis (Beaton & Krug, 1986, Proc. Natl. Acad. Sci. USA 83:6282-6286). NP is also required for elongation of RNA chains (Shapiro & Krug, 1988, J. Virol. 62: 2285-2290) but not for initiation (Honda, et al., 1988, J. Biochem. 104: 1021-1026).

25 NS1 is a major non-structural protein expressed by influenza A viruses in infected cells, whose role in infection is not clear. Studies of viruses carrying temperature-sensitive NS1 alleles point to a regulatory role for NS1 in viral gene-
30 expression and/or replication (Wolstenholme, et al., 1980, J. Virol. 35:1-7; Koennecke, et al., 1981, Virol. 110:16-25; Hatada, et al., 1990, J. Gen. Virol. 71: 1283-1292), which is also consistent with its preferentially nuclear accumulation (Greenspan, et
35 al., 1988, J. Virol. 62: 3020-3026). Its expression

has been shown to interfere with cellular functions in a variety of ways. (Fortes, et al., 1994, EMBO J. 13: 704-712; Qiu & Krug, 1994, J. Virol. 68: 2425-2432; Lu, et al., 1994, Genes Dev. 8: 1817-1828). These effects have been suggested to be mediated through

5 NS1's observed interactions with a variety of RNA's, including single- and double-stranded influenza vRNA (Hatada & Fukuda, 1992, J. Gen. Virol. 73: 3325-3329; Hatada, et al., 1992, J. Gen Virol. 73: 17-25), poly-adenosine RNA (Qiu & Krug, 1994, *supra*), and
10 spliceosomal U6 RNA (Lu, et al., 1994, *supra*). Despite these studies involving the interaction of NS1 with various RNAs, no host proteins that interact with NS1 during infection have previously been identified or characterized.

15 Little is known about host cell functions which contribute to the intracellular replication of influenza viruses, and cellular factors have not been characterized which directly interact with the viral proteins, much less cellular factor/viral interactions
20 that can be used as targets for therapeutic intervention.

3. SUMMARY OF THE INVENTION

The present invention relates to the
25 identification of host cell proteins that interact with viral proteins required for virus replication, and high throughput assays to identify compounds that interfere with the specific interaction between the viral and host cell protein. Interfering compounds
30 that inhibit viral replication can be used therapeutically to treat viral infection.

The invention is based, in part, on the Applicants' discovery of a novel interaction between influenza viral proteins, such as NP and NS1, and
35 human host cell proteins referred to herein as NPI-1,

NPI-2, NPI-3, NPI-4, NPI-5, NPI-6, and NS1I-1, respectively. The host cell proteins such as NPI-1 and NS1I-1 may be accessory proteins required for replication of influenza virus. Compounds that interfere with the binding of the host cell and viral proteins and inhibit viral replication can be useful for treating viral infection in vivo.

4. DESCRIPTION OF THE FIGURES

FIG. 1A and 1B: The interactive trap system, as used in the identification of NP-interacting proteins. FIG. 1A: R100 contains the reporter gene LexAop-LEU2 and a transcriptionally inactive LexA-NP fusion protein (left). Library proteins are synthesized in R100 transformants in media containing galactose. If the library protein does not interact with the LexA-NP fusion protein, then the LEU2 gene is not transcribed (middle). If the library protein does interact with the LexA-NP fusion protein, then the LEU2 gene is transcriptionally active, and the cell forms a colony on leu medium (right). FIG. 1B: The pLexA-NP bait plasmid used in the interactive trap. The coding region of influenza A/PR/8/34 virus nucleoprotein (NP) was subcloned into the *EcoRI* and *Sal I* restriction sites of pEG202. This construction encodes a fusion protein which includes 202 amino acids of LexA and the entire coding region of NP (498 amino acids) separated by 3 amino acids encoded by polylinker sequences derived from the cloning process.

FIG. 2. Nucleotide sequence of NPI-1 cDNA and deduced protein sequence. The coding sequence starts at nucleotide 1 and ends at nucleotide 1581. The 5' terminus of the library clone is indicated by an asterisk. Regions complementary to nested reverse transcription and 5'RACE primers are underlined.

FIG. 3. Comparison of NPI-1 and SRP1. Vertical lines indicate identity; colons and periods indicate conservative changes (Deveraux et al., 1984, Nucl. Acids Res. 12: 387-395). 42 amino acid ARM repeats are aligned vertically according to Peifer et al., 1994, Cell 76: 789-791. For a complete comparison of SRP1 to other ARM repeat containing proteins, see Peifer et al., 1994, *supra*. The ARM consensus sequence is indicated at the bottom; "+" indicates K, R, or H; "-" indicates D or E; "~" indicates a gap. Since other residues are conserved within the repeats of NPI-1 and SRP1, a consensus sequence derived from only these two proteins is also shown.

FIG. 4. GST-NPI-1 binds to NP *in vitro*. GST (lanes 1,5,6) and GST-NPI-1 (lanes 2,3,7,8) were expressed in bacteria and precipitated from cell lysates on glutathione agarose beads. The complexed beads were then incubated with partially purified influenza virus NP and polymerase preparations (Pol/NP) as indicated. Precipitated proteins were fractionated on a 12.5% SDS polyacrylamide gel, and either stained with Coomassie blue (lanes 1 to 3), or immunoblotted using the monoclonal antibody HT103 directed against the viral nucleoprotein (lanes 4 to 8). Unprecipitated Pol/NP was separated in lane 4. M, protein molecular weight markers; *, GST-NPI-1 fusion protein; arrows indicate major fusion protein degradation products.

FIG 5. Immunoblot of total cellular proteins using polyclonal rabbit sera against NPI-1. Total cell lysates and cytoplasmic cell extracts from HeLa and MDBK cell lines were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-NPI-1 sera, and developed by ¹²⁵I-protein A. Each lane contains protein from 1 x 10⁵ cells.

FIG 6. NP is co-immunoprecipitated from influenza A virus infected cells by antisera against NPI-1. Infected HeLa cell proteins were labeled with ³⁵S-methionine and ³⁵S-cysteine, and total cell lysates were made as described in the text. Complexes of NPI-1 and NP were precipitated using anti-NPI-1 sera. Precipitated proteins were then fractionated by SDS-PAGE and detected by autoradiography.

FIGS 7-11. Partial DNA sequences of isolated coding regions of five different proteins that interact with the NP of influenza A, as detected using the interactive trap system in yeast. The proteins whose sequences are provided are as follows:

FIG. 7: Partial nucleotide sequence of NPI-2.

FIG. 8: Partial nucleotide sequence of NPI-3.

FIG. 9: Partial nucleotide sequence of NPI-4.

FIG. 10: Partial nucleotide sequence of NPI-5.

FIG. 11: Partial nucleotide sequence of NPI-6.

Fig. 12. Nucleotide sequence of the NS1I-1 gene and the encoded amino acid sequence of the NS1I-1 protein. The sequence of 2572 bp was determined by dideoxy sequencing of two overlapping clones. The first clone, pK5, was isolated from the yeast library and contains the HeLa cell cDNA comprising nucleotide positions 791 to 2572. The second clone, pRACENS1I-1, resulted from the 5'RACE procedure used to obtain cDNA derived from the 5'-end of NS1I-1 mRNA, and comprises nucleotide positions 1 to 944.

Fig. 13. Northern blot analysis of HeLa cell poly(A)-RNA using an NS1I-1-specific probe.

Fig. 14. Co-precipitation of NS1 protein from extracts of A/WSN/33-infected MDCK cells by GST-NS1I-1 and glutathione sepharose. Monolayers of MDCK cells were either infected with influenza A/WSN/33 virus at an m.o.i. of 10 or mock-infected, and labeled with ³⁵S-methionine and cysteine from 5 to 6 hours p.i.

Proteins were extracted and used for binding to glutathione sepharose coated with GST-NS1I-1 (lanes 3 and 8) or GST-protein (lane 6). As controls, extracts were immunoprecipitated with α -NS1 (lane 2), α -M1 (lane 4), or non-immune serum (lane 5). Proteins were
5 analyzed by SDS gel electrophoresis and fluorography. Aliquots of the total extracts corresponding to 10% used for the glutathione agarose precipitations are shown (lanes 1 and 7). The positions of virus
10 proteins and molecular weight markers are indicated to the left.

Fig. 15. GST-NS1I-1 co-precipitates NS1 proteins of influenza A and B virus strains. Extracts of ³⁵S-labeled MDCK cells infected with the influenza viruses A/duck/Alberta/76 (Panel A), A/turkey/Oregon
15 (Panel B), A/Beijing/32/92 (Panel C), A/Berkeley/1/68 (Panel D), and B/Lee/40 (Panel E) were prepared and used in precipitations of viral proteins by glutathione-sepharose coated with GST-NS1I-1 (lanes "GST-K5") or GST-protein (lanes "GST") as described in
20 Fig. 14. In addition, viral proteins were immunoprecipitated using α -NS1-, α -M1- or non-immune serum (lanes " α -NS1", " α -M1", "NI", respectively). Analysis was by SDS gel electrophoresis and
25 fluorography. Aliquots of the total extracts corresponding to 10% (Panels C and E) or 6.7% (Panels A, B, and D), respectively, are also shown (lanes "T"). The positions of viral proteins are indicated to the right.

30 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification of host cellular proteins that interact with viral proteins important to viral replication and infection; the identification of compounds that
35 interfere with the specific interaction of the host

cell and viral proteins; and the evaluation and use of such compounds as antivirals in the treatment of viral infections in animals, including humans.

The invention is described in this section and in the examples, below for the identification and
5 inhibition of interactions between human host cell proteins and influenza viral proteins. For clarity of discussion, particular detail is provided for the isolation of two particular host cell proteins. The first such protein is nucleoprotein interactor 1
10 (NPI-1), a human cell protein that interacts with the influenza virus NP protein. The NPI-1 gene and protein, and the protein's interaction with NP protein are described in detail in the example in Section 6, below. Other host cell proteins which interact with
15 the NP protein include, but are not limited to, NPI-2, NPI-3, NPI-4, NPI-5, and NPI-6, and are also described, below. Since the interactions between NP and the NPI-1 through NPI-6 host cell proteins have never before been identified, they provide novel
20 targets for antiviral treatment and serve as excellent models for detailing the aspects of the invention. However, the principles may be analogously applied to the identification of other host cell proteins that interact with any of the four influenza virus proteins
25 (PA, PB1, PB2, in addition to NP) required for viral RNA replication.

Particular detail is also provided in the example in Section 7, below, for the identification of nonstructural protein 1 interactor 1 (NS1I-1). NS1I-1
30 is a human cell protein that interacts with the influenza virus NS1 protein. This interaction also has never before been described, and, therefore, provides yet another novel target for antiviral treatment. The present invention also contemplates
35 identifying interactions between host cell proteins

and other viral proteins (in addition to NS₁) required for infection, such as, in the case of influenza virus, NS₂, HA, NA, M₁, and M₂ proteins.

The principles may also be analogously applied to other RNA viruses, including but not limited to paramyxoviruses, such as parainfluenza viruses, measles viruses, respiratory syncytial virus, bunyviruses, arena viruses, the orthomyxo-like insect virus called Dhori, etc. The host cell proteins so identified may include completely novel proteins, or previously described proteins that have not yet been shown to interact with viral proteins. Any method suitable for detecting protein-protein interactions may be employed for identifying novel viral-host protein interactions, and are considered within the scope of the present invention. For example, some traditional methods are co-immunoprecipitation, crosslinking and copurification through gradients or chromatographic columns. Newer methods result in the simultaneous identification of the genes coding for the protein interacting with a target protein. These methods include probing expression libraries with labeled target protein in a manner similar to antibody probing of λ gt11 libraries. One such method which detects protein interactions *in vivo*, the yeast interactive trap system, was successfully used as described herein to identify the host cell proteins NPI-1 through NPI-6, and NS1I-1, described herein, and is described in detail for illustration only and not by way of limitation.

The host cell/viral protein interactions identified are considered targets for antiviral intervention. Assays, such as the ones described herein, can be used to identify compounds that interfere with such interactions. The compounds so identified which inhibit virus infection, replication,

assembly, or release can be used as antivirals. In accordance with the invention, a given compound found to inhibit one virus may be tested for antiviral activity against a wide range of different viruses that have analogous dependencies on host cell

5 proteins, including but not limited to paramyxoviruses, such as parainfluenza viruses, measles viruses, respiratory syncytial virus, bunyviruses, arena viruses, the orthomyxo-like insect virus called Dhorì, etc.

10 Elucidation of the roles of the interacting proteins will lead to identifying other viruses as targets for intervention. For example, we have found that NPI-1 is important to the import of viral nucleic acid-protein complex into the nucleus of the host

15 cell. Therefore, methods described below that disrupt this process, through interfering with the activity of NPI-1, for example, may be effective in treating viruses with nuclear phases, in addition to those viruses listed above. Such additional viruses

20 include, but are not limited to, human immunodeficiency virus (HIV), members of the herpes virus family, and adenoviruses.

The various aspects of the invention are described in the subsections below with specific

25 reference to host cell proteins that interact with NP (NPI-1 through NPI-6) and NS1 (NS1I-1), with particular emphasis on NPI-1; however, the invention is not limited to NPI-1 and encompasses any viral/host cell protein interactions as targets for therapeutic

30 intervention.

5.1. IDENTIFICATION OF HOST CELL PROTEINS THAT INTERACT WITH VIRAL PROTEINS REQUIRED FOR REPLICATION

The previously unidentified gene for the host cell protein NPI-1 was cloned based on its ability to interact with the influenza A virus NP. The NPI-1 is the human homolog of the yeast protein SRP1. Interaction of NPI-1 and NP was demonstrated in yeast
5 by the interactive trap system; *in vitro* coprecipitation of the NP with a bacterially expressed NPI-1 protein; and in infected cell extracts by coprecipitation of the NP with NPI-1, using anti-NPI-1 sera. The demonstration of this previously unknown
10 interaction is illustrated in the working examples (see Section 6, *infra*). The data generated indicate that NPI-1 plays a role in the replication of influenza A viruses. NPI-1 is the first cellular protein characterized which interacts with a protein
15 encoded by influenza viruses. This role, therefore, makes the inhibition of the NP-NPI-1 interaction an excellent target for antiviral therapy. It has not yet been demonstrated at what stage in the replication cycle NPI-1 functions. The NPI-1 could affect any of
20 a number of NP functions which may include: (1) movement of the ribonucleoprotein complex (RNP) to the nucleus during viral entry; (2) vRNA synthesis, including antitermination and elongation; (3) mRNA synthesis, including elongation, polyadenylation, and
25 transport to the cytoplasm; and (4) exit of the RNP from the nucleus during virion assembly.

The fact that both NPI-1 and SRP1 interact with proteins involved in RNA synthesis implies that there may be fundamental similarities between cellular
30 DNA-dependent transcription and influenza viral RNA-dependent RNA synthesis. Cellular factors, like NPI-1, may be shared by the viral and the cellular RNA synthesis machinery to perform similar functions. In addition, the NPI-1 may tether the viral RNP to areas
35 of the nuclear matrix where splicing and

polyadenylation of mRNA occur. It should be noted that although NPI-1 was isolated from HeLa cells, this cell line is not productively infected by influenza A virus. However, HeLa cells synthesize influenza viral RNAs and proteins (see Fig. 6, lane 3), and have
5 previously been used to examine viral RNA synthesis (Beaton & Krug, 1986, *supra*).

The viral NP exists in two forms in the infected cell. One form is associated with ribonucleoprotein complexes (RNP), and the other is a free form
10 (Shapiro & Krug, 1988, *supra*). Pol/NP preparations used in coprecipitation experiments with NPI-1 were purified over cesium chloride/glycerol gradients (Honda et al., 1988, *supra*), which dissociate and purify virion proteins away from vRNA. The NP but not
15 the polymerase proteins were detected on Coomassie stained gels in this experiment (Fig 4, lane 3); however, coprecipitation of the viral polymerase proteins was not rigorously tested by immunoblot experiments. Only the NP was coprecipitated from
20 infected HeLa cell extracts (Fig. 6) suggesting that it is free NP which is bound by NPI-1.

Only one host factor has been assigned a definitive function in the replication process of a negative strand RNA virus. The cellular casein kinase
25 II has been shown to phosphorylate the phosphoprotein P of the vesicular stomatitis virus (VSV) RNA-dependent RNA polymerase. This is a step which appears to be required in order to activate the viral polymerase (Barik and Banerjee, 1992, *Proc. Natl.*
30 *Acad. Sci. USA* 89: 6570-6574; Barik and Banerjee, 1992, *J. Virol.* 66: 1109-1118).

NPI-1 and SRP1 are 50% identical and 81% conserved at the amino acid level. This is a very high degree of conservation between proteins belonging
35 to organisms as distantly related as humans and yeast,

and suggests that the NPI-1/SRP1 performs a very basic function in the cell. NPI-1 and SRP1 have eight internal repeats, each of approximately 42-amino acids (Fig. 3). This repeat, termed the ARM motif, was originally identified in the *Drosophila* segment polarity gene *armadillo* (Riggelman, et al., 1989, Genes Dev. 3: 96-113), and it has been identified in a number of other proteins including β -catenin, plakoglobin, p120, APC and smGDS (Peifer et al., 1994, *supra*, and references therein). Several ARM proteins are associated with cell adhesion structures. *Armadillo* and its homologues bind to the C-terminal cytoplasmic tail of cadherins, a calcium-dependent class of cell adhesion molecules (CAMs), linking the CAMs to the underlying cytoskeleton at cell-cell junctions (McCrea, et al., 1991, Science 254: 1359-1361). In contrast to the *armadillo* protein, SRP1 and NPI-1 appear to be localized to the nucleus. If NPI-1, like SRP1 (Yano, et al., 1992, Mol. Cell. Biol. 12: 5640-5651), is associated with the nuclear membrane, it is possible that NPI-1 functions to tether viral RNP to the nuclear membranes (Jackson, et al., 1982, Nature 296: 366-368). It should be noted that NPI-1 may be related to (or identical with) a nuclear protein that has been found to be involved in V(D)J recombination (Cuomo et al., 1994, Meeting abstract F015, Keystone Symposium on Recombination).

The carboxyl terminal 265 amino acids of the NPI-1, which were sufficient for interaction with the viral NP, contain four and one-half ARM repeats. Individual repeats, in general, are approximately 30 % identical with the ARM consensus sequence. This is consistent with the degree of conservation in ARM repeats of other proteins (Peifer et al., 1994, *supra*).

35

Using the same interactive trap system in yeast, five additional DNA sequences were isolated which partially encode proteins that interact with the NP of influenza A virus. Also, using this system, a DNA sequence encoding the NS1I-1 protein was identified
5 based the interaction between NS1I-1 and the NS1 protein of influenza A virus. This protein is the human homolog of porcine 17 β -estradiol dehydrogenase. Several proteins with a dehydrogenase function have recently been shown to be involved in post-
10 transcriptional events of gene expression (Hentze, 1994, Trends Biochem. Sci. 19: 101-103). This supports an important functional role for the NS1I-1 interaction during the viral life cycle. The various proteins so identified are listed in Table I.

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TABLE I
INTERACTING HOST CELL PROTEINS

5	Host Cell Proteins		
	FIG.	Comments	
10	NPI-1	FIG. 2	New protein sequence, homologous to SRP1 of Yeast
	NPI-2	FIG. 7	Identical to sequences of hnRNP C proteins (Lahiri & Thomas, 1986, Nucl. Acids Res. 14: 4077-4094)
15	NPI-3	FIG. 8	New protein sequence
	NPI-4	FIG. 9	New protein sequence
	NPI-5	FIG. 10	New protein sequence
	NPI-6	FIG. 11	New protein sequence
	NS1I-1	FIG. 12.	New protein sequence, homologous to porcine 17 β -estradiol dehydrogenase
20	Note: Recently performed searches of Genbank have revealed that subsequent to Applicants' identification of NPI-3, NPI-4, and NPI-5, these sequences were described by other groups and designated Rchl, PCA, and BAT1, respectively.		

The coding sequence for NPI-2 is identical to sequences coding for the previously identified hnRNP C proteins (Lahiri & Thomas, 1986, *supra*). The NPI-3, NPI-4, NPI-5, and NPI-6 coding sequences were unknown prior their identification by Applicants. The NS1I-1 gene is also novel, as explained in detail in the example in Section 7, below.

The invention contemplates, in addition to the DNA sequences disclosed herein, 1) any DNA sequence that encodes the same amino acid sequence as encoded by the DNA sequences shown in Figures 2 and 7-12; 2) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein (see Figs. 2 and

7-12) under highly stringent conditions, e.g., washing
in 0.1xSSC/0.1% SDS at 68°C (Ausubel, F.M. et al.,
eds., 1989, Current Protocols in Molecular Biology,
Vol. I, Green Publishing Associates, Inc., and John
Wiley & sons, Inc., New York, at p. 2.10.3) and
5 encodes a functionally equivalent gene product; and/or
3) any DNA sequence that hybridizes to the complement
of the coding sequences disclosed herein (see Figs. 2
and 7-12) under less stringent conditions, such as
moderately stringent conditions, e.g., washing in
10 0.2xSSC/0.1% SDS at 42°C (Ausubel, et al., 1989,
supra), yet which still encodes a functionally
equivalent gene product.

The invention also encompasses 1) DNA vectors
that contain any of the coding sequences disclosed
15 herein (see Figs. 2 and 7-12), and/or their
complements (i.e., antisense); 2) DNA expression
vectors that contain any of the coding sequences
disclosed herein (see Figs. 2 and 7-12), and/or their
complements (i.e., antisense), operatively associated
20 with a regulatory element that directs the expression
of the coding and/or antisense sequences; and 3)
genetically engineered host cells that contain any of
the coding sequences disclosed herein (see Figs. 2 and
7-12), and/or their complements (i.e., antisense),
25 operatively associated with a regulatory element that
directs the expression of the coding and/or antisense
sequences in the host cell. Regulatory element
includes but is not limited to inducible and non-
inducible promoters, enhancers, operators and other
30 elements known to those skilled in the art that drive
and regulate expression. The invention includes
fragments of any of the DNA sequences disclosed
herein.

Once the host cell proteins are obtained, they
35 can be used to detect interactions with proteins from

other viruses, in accordance with the invention. The following description is provided to illustrate this approach and not by way of limitation. Influenza B virus ribonucleoprotein complex was isolated and using a Western immunoblot assay, it was found that the
5 cellular NPI-1 was associated with this complex. This result indicates that NPI-1, isolated based on its interaction with influenza A virus NP, also interacts with influenza B virus NP. Thus, compounds that inhibit NP-NPI-1 interactions in influenza A virus and
10 thereby inhibit influenza A viral infection should be similarly effective as antivirals against influenza B virus.

Host cell genes that are homologous to those identified herein may be obtained by several methods.
15 In some cases, different host cell proteins that share the property of interacting with the same viral protein, e.g. influenza A virus NP, may also share genetic homology. Thus, the genes identified through the interactive trap selection may be homologous to
20 one another.

Once a host cell gene is identified in accordance with the invention, any homologous gene may be obtained using cloning methods well known to those skilled in the art, including but not limited to the
25 use of appropriate probes to detect the homologous genes within an appropriate cDNA or gDNA (genomic DNA) library. (See, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, which is incorporated by
30 reference herein its entirety. This method is especially useful for obtaining proteins that may not share the property of binding to the same viral protein, but may nonetheless be genetically
35 homologous.

Such homologous proteins may interact with proteins of viruses other than the virus used in the interactive trap. For example, a host cell gene whose product was detected through its interaction with an influenza A viral protein may be homologous to another
5 gene whose product does not interact with influenza A virus, but which does interact with influenza B viral protein. To optimize the detection of such a homologous gene, cDNA libraries may be constructed from cells infected with a virus of interest. Besides
10 influenza B virus, this procedure may be applied analogously to other viruses as well, including but not limited to paramyxoviruses, such as parainfluenza viruses, measles viruses, respiratory syncytial virus, bunyviruses, arena viruses, the orthomyxo-like insect
15 virus called Dhori, as well as human immunodeficiency virus (HIV), members of the herpes virus family, and adenoviruses.

20 5.2. SCREENING ASSAYS FOR COMPOUNDS THAT
INTERFERE WITH THE INTERACTION OF
HOST CELL AND VIRAL PROTEINS
REQUIRED FOR VIRAL REPLICATION

The host cell protein and the viral protein which interact and bind are sometimes referred to herein as "binding partners". This term also includes peptide
25 fragments, produced as described in the subsections below, comprising the binding domain of each respective protein. Any of a number of assay systems may be utilized to test compounds for their ability to interfere with the interaction of the binding
30 partners. However, rapid high throughput assays for screening large numbers of compounds, including but not limited to ligands (natural or synthetic), peptides, or small organic molecules are preferred. Compounds that are so identified to interfere with the
35 interaction of the binding partners should be further

evaluated for antiviral activity in cell based assays, animal model systems and in patients as described herein.

The basic principle of the assay systems used to identify compounds that interfere with the interaction
5 between the viral and host cell proteins involves preparing a reaction mixture containing the viral protein and the host cell protein under conditions and for a time sufficient to allow the two proteins to interact and bind, thus forming a complex. In order
10 to test a compound for inhibitory activity, the reaction is conducted in the presence and absence of the test compound, i.e., the test compound may be initially included in the reaction mixture, or added at a time subsequent to the addition of the viral and
15 host cell protein; controls are incubated without the test compound or with a placebo. The formation of any complexes between the viral protein and the host cell protein is then detected. The formation of a complex in the control reaction, but not in the reaction
20 mixture containing the test compound indicates that the compound interferes with the interaction of the viral protein and host cell protein.

The assay components and various formats that may be utilized are described in the subsections below.
25

5.2.1. ASSAY COMPONENTS

The host cell protein and viral protein binding partners used as components in the assay may be derived from natural sources, e.g., purified from
30 cells and virus, respectively, using protein separation techniques well known in the art; produced by recombinant DNA technology using techniques known in the art (see e.g., Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor
35 Laboratories Press, Cold Spring Harbor, N.Y.); and/or

chemically synthesized in whole or in part using techniques known in the art; e.g., peptides can be synthesized by solid phase techniques, cleaved from the resin and purified by preparative high performance liquid chromatography (see, e.g., Creighton, 1983, 5 Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing; e.g., using the Edman degradation procedure (see e.g., Creighton, 1983, 10 *supra* at pp. 34-49).

The peptide fragments should be produced to correspond to the binding domains of the respective proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the 15 protein's binding site. These methods include but are not limited to mutagenesis of one of the genes encoding the protein and screening for disruption of binding in a co-immunoprecipitation assay, or mutagenesis of the host cell gene and selecting for 20 resistance to viral infection. Compensating mutations in the viral gene can be selected which allow for viral growth in this mutant host. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of 25 the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in section 5.2.2. *infra*, and allowed to interact with and bind to its labeled binding partner, which has been treated with a 30 proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene for the protein is 35 obtained, short gene segments can be engineered to

express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

Whether produced by molecular cloning methods or by chemical synthetic methods, the amino acid sequence
5 of the binding partners which may be used in the assays of the invention need not be identical to the reported sequence of the genes encoding them. The binding partners may comprise altered sequences in which amino acid residues are deleted, added, or
10 substituted resulting in a functionally equivalent product.

For example, functionally equivalent amino acid residues may be substituted for residues within the sequence resulting in a change of sequence. Such
15 substitutes may be selected from other members of the class to which the amino acid belongs; e.g., the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; the polar neutral amino
20 acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; the positively charged (basic) amino acids include arginine, lysine, and histidine; the negatively charged (acidic) amino acids include aspartic and glutamic acid.

25 One of the binding partners used in the assay system should be labeled, either directly or indirectly, to facilitate detection of a complex formed between the viral and host cell proteins. Any of a variety of suitable labeling systems may be used
30 including but not limited to radioisotopes such as ¹²⁵I; enzyme labelling systems that generate a detectable colorimetric signal or light when exposed to substrate; and fluorescent labels.

Where recombinant DNA technology is used to
35 produce the viral and host cell binding partners of

the assay it may be advantageous to engineer fusion proteins that can facilitate labeling, immobilization and/or detection. For example, the coding sequence of the viral or host cell protein can be fused to that of a heterologous protein that has enzyme activity or serves as an enzyme substrate in order to facilitate labeling and detection. The fusion constructs should be designed so that the heterologous component of the fusion product does not interfere with binding of the host cell and viral protein.

Indirect labeling involves the use of a third protein, such as a labeled antibody, which specifically binds to one of the binding partners, i.e., either the host cell protein or viral protein used. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library.

For the production of antibodies, various host animals may be immunized by injection with the host cell protein or the viral protein, or a portion thereof. Such host animals may include but are not limited to rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the

hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72, Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma
5 technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al.,
10 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be
15 used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies specific to one of the binding partners.

Antibody fragments which recognize specific
20 epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the
25 disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

30

5.2.2. ASSAY FORMATS

The assay can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring one of the binding partners onto a solid
35 phase and detecting complexes anchored on the solid

phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For
5 example, test compounds that interfere with the interaction between the binding partners, e.g., by competition, can be identified by conducting the reaction in the presence of the test substance; i.e.,
10 by adding the test substance to the reaction mixture prior to or simultaneously with the viral protein and host cell protein. On the other hand, test compounds that disrupt preformed complexes, e.g. compounds with higher binding constants that displace one of the
15 binding partners from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, one binding partner, e.g., either the viral protein or the host
20 cell protein, is anchored onto a solid surface, and its binding partner, which is not anchored, is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or
25 covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody specific for the protein may be used to anchor the protein to the solid surface.
30 The surfaces may be prepared in advance and stored.

In order to conduct the assay, the binding partner of the immobilized species is added to the coated surface with or without the test compound. After the reaction is complete, unreacted components
35 are removed (e.g., by washing) and any complexes

formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the binding partner was pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the binding partner is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the binding partner (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one binding partner to anchor any complexes formed in solution, and a labeled antibody specific for the other binding partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the host cell and viral protein is prepared in which one of the binding partners is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the

binding partners from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt the viral protein-host cell protein interaction can be identified.

5 For example, in a particular embodiment for NPI-1, NPI-1 can be prepared for immobilization using recombinant DNA techniques described in section 5.2.1., *supra*. Its coding region can be fused to the glutathione-S-transferase (GST) gene using the fusion
10 vector pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. NP can be purified and used to raise a monoclonal antibody, specific for NP, using methods routinely practiced in the art and described above.
15 This antibody can be labeled with the radioactive isotope ^{125}I , for example, by methods routinely practiced in the art. In a heterogeneous assay, *e.g.*, the GST-NPI-1 fusion protein can be anchored to glutathione-agarose beads. NP can then be added in
20 the presence or absence of the test compound in a manner that allows NP to interact with and bind to the NPI-1 portion of the fusion protein. After the test compound is added, unbound material can be washed away, and the NP-specific labeled monoclonal antibody
25 can be added to the system and allowed to bind to the complexed binding partners. The interaction between NP and NPI-1 can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of
30 the interaction by the test compound will result in a decrease in measured radioactivity.

 Alternatively, the GST-NPI-1 fusion protein and NP can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test
35 compound can be added either during or after the

binding partners are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the binding partner interaction can be detected by measuring the
5 radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of NP and NPI-1, respectively, in place of one or both of
10 the full length proteins. These binding domains can be identified, as described in section 5.2.1., *supra*. For example, and not by way of limitation, NPI-1 can be anchored to a solid material as described above in this section by making a GST-NPI-1 fusion protein and
15 allowing it to bind to glutathione agarose beads. NP can be labeled with a radioactive isotope, such as ³⁵S, and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-NPI-1 fusion protein and allowed to bind. After
20 washing away unbound peptides, labeled bound material, representing the NP binding domain, can be eluted, purified, and analyzed for amino acid sequence by methods described in section 5.2.1., *supra*. Peptides so identified can be produced synthetically or fused
25 to appropriate facilitative proteins using recombinant DNA technology, as described in section 5.2.1., *supra*.

In accordance with the invention, a given compound found to inhibit one virus may be tested for general antiviral activity against a wide range of
30 different viruses that have analogous dependencies on host cell proteins. For example, and not by way of limitation, a compound which inhibits the interaction of influenza virus NP with NPI-1 by binding to the NP binding site can be tested, according to the assays
35 described in section 5.3. *infra*, against other

viruses, particularly those which have similar proteins, e.g., parainfluenza viruses.

5.3. ASSAYS FOR ANTIVIRAL ACTIVITY

Any of the inhibitory compounds which are
5 identified in the foregoing assay systems may be tested for antiviral activity.

5.3.1. VIRAL GROWTH ASSAYS

The ability of an inhibitor identified in the
10 foregoing assay systems to prevent viral growth can be assayed by plaque formation or by other indices of viral growth, such as the TCID₅₀ or growth in the allantois of the chick embryo. In these assays, an appropriate cell line or embryonated eggs are infected
15 with wild-type influenza virus, and the test compound is added to the tissue culture medium either at or after the time of infection. The effect of the test compound is scored by quantitation of viral particle formation as indicated by hemagglutinin (HA) titers
20 measured in the supernatants of infected cells or in the allantoic fluids of infected embryonated eggs; by the presence of viral plaques; or, in cases where a plaque phenotype is not present, by an index such as the TCID₅₀ or growth in the allantois of the chick
25 embryo, or with a hemagglutination assay.

An inhibitor can be scored by the ability of a test compound to depress the HA titer or plaque formation, or to reduce the cytopathic effect in virus-infected cells or the allantois of the chick
30 embryo, or by its ability to reduce viral particle formation as measured in a hemagglutination assay.

5.3.2 ANIMAL MODEL ASSAYS

The ability of an inhibitor to prevent
35 replication of influenza virus can be assayed in

animal models that are natural or adapted hosts for influenza. Such animals may include mammals such as pigs, ferrets, mice, monkeys, horses, and primates, or birds. As described in detail in Section 5.5 *infra*, such animal models can be used to determine the LD₅₀ and the ED₅₀ in animal subjects, and such data can be used to derive the therapeutic index for the inhibitor of the viral/host cell protein interaction.

5.4. INHIBITORY COMPOUNDS

Inhibitory compounds identified in the foregoing screening assays which may be used in accordance with the invention may include but are not limited to small organic molecules, peptides and antibodies.

For example, peptides having an amino acid sequence corresponding to the domain of the host cell protein that binds to the viral protein may be used to compete with the native viral protein and, therefore, may be useful as inhibitors in accordance with the invention. Similarly, peptides having an amino acid sequence corresponding to the domain of the viral protein that binds to the host cell protein may be used. Such peptides may be synthesized chemically or produced via recombinant DNA technology using methods well known in the art (*e.g.*, see Creighton, 1983, *supra*; and Sambrook et al., 1989, *supra*). Lipofectin or liposomes may be used to deliver the peptides to cells.

Alternatively, antibodies that are both specific for the binding domains of either the host cell or viral proteins and interfere with their interaction may be used. Such antibodies may be generated using standard techniques described in Section 5.2.1., *supra*, against the proteins themselves or against peptides corresponding to the binding domains of the proteins. Such antibodies include but are not limited

to polyclonal, monoclonal, Fab fragments, single chain antibodies, chimeric antibodies, etc. Where whole antibodies are used, internalizing antibodies are preferred. However, lipofectin may be used to deliver the antibody or a fragment of the Fab region which
5 binds to the viral or host cell protein epitope into cells. Where fragments of the antibody are used, the smallest inhibitory fragment which binds to the target protein's binding domain is preferred.

10 5.5. PHARMACEUTICAL PREPARATIONS
 AND METHODS OF ADMINISTRATION

 The identified compounds that inhibit viral replication can be administered to a patient at therapeutically effective doses to treat viral
15 infection. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of viral infection.

 Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical
20 procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects
25 is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that
30 targets such compounds to the site of infection in order to minimize damage to uninfected cells and reduce side effects.

 The data obtained from the cell culture assays and animal studies can be used in formulating a range
35 of dosage for use in humans. The dosage of such

compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any

5 compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50

10 (i.e., the concentration of the test compound which achieves a half-maximal infection, or a half-maximal inhibition) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be

15 measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically

20 acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal,

25 parenteral or rectal administration.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser,

30 with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve

35 to deliver a metered amount. Capsules and cartridges

of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycollate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

35

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection
5 or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous
10 vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

15 The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described
20 previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be
25 formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in
30 a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions
35 for administration.

6. EXAMPLE: THE IDENTIFICATION OF NPI-1
AND ITS INTERACTION WITH
INFLUENZA NUCLEOPROTEIN

The yeast interactive trap system was used to identify a cellular protein which interacts with the nucleoprotein of influenza A viruses. This protein, 5 nucleoprotein interactor 1 (NPI-1) is the human homologue of the yeast protein SRP1. SRP1 was previously identified as a suppressor of temperature-sensitive RNA polymerase I mutations (Yano, et. al., 1992, Mol. Cell. Biol. 12:5640-5651). A full length 10 cDNA clone of NPI-1 was generated from HeLa cell poly A+ RNA. The viral NP, which had been partially purified from influenza A/PR/8/34 virus-infected embryonated eggs, could be coprecipitated from solution by glutathione agarose beads complexed with a 15 bacterially expressed glutathione-S-transferase (GST)-NPI-1 fusion protein, confirming the results of the yeast genetic system. Antisera raised against NPI-1 identified a 65 kDa polypeptide from total cellular extracts of both HeLa and MDBK cells. In addition, 20 the viral nucleoprotein was co-immunoprecipitated from influenza A/WSN/33 virus-infected HeLa cells by antisera directed against NPI-1, demonstrating an interaction of these two proteins in infected cells, and suggesting that NPI-1 plays a role during 25 influenza virus replication.

6.1. MATERIALS AND METHODS

6.1.1. YEAST, BACTERIA AND PLASMIDS

30 Yeast strain EGY48 (*Mata trp1 ura3 his3 LEU2::pLEXAop6-LEU2*) (Zervos et al., 1993, Cell 72: 222-232) and plasmids pEG202, pSH18-34, and pRFHM1 and the HeLa cell cDNA library constructed in pJG4-5 (Gyuris et al., 1993, Cell 75: 791-803) were 35

previously described. Similar versions of these plasmids and this yeast host strain are available commercially from Clontech as part of a two fusion protein system. pLexA-NP was constructed by subcloning the cDNA of influenza A/PR/8/34 NP as a
5 LexA translational fusion gene into pEG202 (Fig. 1). Yeast strains constructed as part of these studies are described in Table 2. *Escherichia coli* MH3 (*trpC araD lacX hsdR galU galK*) and W31005 were previously described (Hall et al., 1984, Cell 36: 1057-1065).

10

6.1.2. SELECTION OF NP INTERACTORS

An interactive trap selection was performed essentially as has been previously described (Gyuris, et al., 1993, *supra*; Zervos, et al., 1993, *supra*).
15 Strain R100 was transformed by the HeLa cDNA library using the lithium acetate method (Ito, et al., 1983, J. Bacteriol. 153: 163-168). 2×10^6 primary yeast transformants were selected on twelve 25 x 25 cm² his⁻ trp⁻-glucose plates, pooled and stored at -70°C.
20 Library transformants were selected for leu⁺ phenotype on his⁻leu⁻-galactose plates; the efficiency of plating was approximately 10^4 leu⁺ colonies per galactose⁺ colony. Plasmid DNA was isolated from leu⁺ library transformants as described by Hoffman and Winston
25 (Hoffman & Winston, 1987, Gene 57: 267-272) and introduced into MH3 cells by electroporation. Library plasmids were selected by plating the transformation mix on 1xA+amp+glucose plates (Miller, 1972, Experiments in Molecular Genetics, Cold Spring Harbor
30 Laboratory Press, Cold Spring Harbor, NY).

cDNAs were analyzed by checking the specificity of interaction with the NP. Each isolated plasmid was introduced into strains R101 and R102. These strains harbor pSH18-34, a reporter plasmid encoding
35

β -galactosidase with a GAL1 promoter transcriptionally controlled from upstream LexA binding sites. Strain R102 was used as a negative control for NP-specificity of cloned cDNAs. It contains pRFHM1, which encodes LexA fused to a transcriptionally inert fragment of the *Drosophila melanogaster* bicoid protein. β -Galactosidase activity was assayed on nitrocellulose replicas of the colonies by freeze fracturing the cells and incubating in buffer containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) (Miller, 1972, *supra*). Plasmids which conferred both a leu+ and β -gal+ phenotypes in the presence of pLexA-NP but not in the presence of pRFHM1 were saved for further study.

6.1.3. CLONING OF THE 5' TERMINUS OF NPI-1

The 5' terminus of NPI-1 was cloned by rapid amplification of cDNA ends ("RACE") by the method of Frohman (Frohman, 1990, in PCR Protocols: A Guide to Methods and Applications, Innis et. al., eds., Academic Press Inc., San Diego, p. 28-38; Frohman, et al., 1988, Proc. Natl. Acad. Sci. USA 85: 8998-9002). Reverse transcription of 1 μ g of poly A+ HeLa cell RNA was performed using the NPI-1 specific oligonucleotide 5'GCAAAGCAGGAGAAACCAC3'. First strand cDNA was tailed with dCTP by terminal transferase. PCR amplification of the reverse transcription product was performed with the nested NPI-1 primer 5'GGGTCCATCTGATAGATATGAGAG3' and the 5' RACE anchor primer 5'CUACUACUACUAGGCCACGCGTCGACTACTACGGGIIGGGIIGGGIIG3' (Gibco/BRL). The PCR product was subcloned into pGEM-T (Promega) and was sequenced by standard protocols. 5'RACE products from three independent experiments were cloned and sequenced in order to avoid errors introduced by PCR.

6.1.4. BACTERIAL EXPRESSION AND PURIFICATION OF GST-NPI-1

The NPI-1 cDNA derived from a HeLa cDNA library was subcloned into the *EcoRI* and *XhoI* restriction endonuclease sites of the glutathione-S-transferase fusion vector pGEX-5X-1 (Pharmacia) to generate the plasmid pGST-NPI-1. Protein was induced from bacterial expression plasmids in W31005 cells with isopropyl- β -D-galactopyranoside according to standard protocols (Smith & Johnson, 1988, Gene 67: 31-40). Bacteria were pelleted 4 h after induction, washed in ice cold phosphate buffered saline (PBS), and resuspended in one-tenth culture volume PBS+1% Triton X-100. Bacteria were lysed on ice with four 15 s pulses in a Raytheon sonicator at an output setting of 1 amp. Insoluble material was pelleted at 50,000xg for 30 min in a Beckman TL-100.3 rotor.

GST-NPI-1 and GST were purified from bacterial lysates on glutathione-agarose beads (Sigma Chemical Corp.). Beads were swelled according to the manufacturer's instructions and equilibrated in PBS. Typical binding reactions were done in 500 μ l of PBS/0.1% Triton X-100, and included 50 μ l bacterial lysate and 10 μ l of a 50% slurry of glutathione-agarose beads. Binding reactions were incubated for 5 min at room temperature on a rotating wheel. Beads were collected by centrifugation for 5 s in a microfuge, and were washed three times in PBS.

6.1.5. NP BINDING ASSAY

To assay binding of NP to GST-NPI-1/beam complexes typical reactions were performed in 500 μ l of ice cold PBS+0.05% Nonidet P-40 and contained washed GST-NP1-1/beam complexes and 10 μ g partially purified influenza virus polymerase and nucleoprotein preparations (Pol/NP). Virus was prepared from

embryonated eggs infected by influenza A/PR/8/34 virus and POL/NP preparations were purified as previously described (Enami, et al., 1990, Proc. Natl. Acad. Sci. USA 87: 3802-3805; Parvin, et al., 1989, J. Virol. 63: 5142-5152). NP was bound for 1 h at 4°C on a rotating wheel. Beads were collected by centrifugation for 5 s in a microfuge, and were washed three times in PBS+0.05% NP-40. Washed beads were resuspended in 50 µl SDS sample buffer (Sambrook, et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories Press, Cold Spring Harbor, NY), boiled for 5 min, and pelleted in a microfuge. 10 µl of each supernatant was separated by electrophoresis on a 12.5% SDS-polyacrylamide gel. Gels were either stained with Coomassie blue or processed for immunoblot analysis. NP was detected by immunoblotting with the monoclonal antibody HT103.

6.1.6. ANTISERA AND IMMUNOBLOTTING

Polyclonal rabbit antisera against NPI-1 was generated by immunization of a female NZY Rabbit (Buckshire Farms) with 200 µg of purified GST-NPI-1 in complete Freund's adjuvant, followed by two boosts of 100 µg in incomplete Freund's adjuvant at three week intervals. The specificity of antisera was demonstrated by immunoblot analysis of GST-NPI-1 in bacterial lysates. Immunoblots were performed by standard methods (Harlow and Lane, 1998, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratories Press, Cold Spring Harbor, NY). Sera were used at a dilution of 1:1000.

6.1.7. VIRUSES AND CELLS

Total cell lysates from HeLa and MDBK cells were generated by direct lysing of cells in SDS-sample buffer, followed by shearing of chromosomal DNA by

passage through a 21 ga. syringe. Cytoplasmic extracts were generated by lysing cells in ice cold NP-40 lysis buffer (10 mM Tris-Cl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 1 mM DTT; 1% Nonidet P-40; 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride-hydrochloride (Pefabloc)). After 10 min on ice nuclei were removed by centrifugation. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and visualized by immunoblotting.

To generate infected cell lysates containing metabolically labeled proteins 4×10^6 HeLa cells were infected with influenza A/WSN/33 virus at a multiplicity of 10 for 45 min at 37°C. Infection was allowed to proceed in DMEM + 0.1% BSA for 5 h at which time cells were labeled with 50 μ Ci 35 S-methionine + 50 μ Ci 35 S-cystine in MEM-cys-met for 1 h. Extracts were prepared by resuspending infected cells in 650 μ l ice cold NP-40 lysis buffer followed by two 15 s pulses in a Raytheon sonicator to disrupt nuclei. Insoluble cell debris was removed by centrifugation at 100,000xg in a TL-100.3 Beckman rotor. 5 μ l anti-NPI-1 sera was incubated on ice for 1 h with 100 μ l infected cell lysates. Immune complexes were precipitated from solution by incubation with Sepharose-4B linked protein G beads (Sigma) for 1 h. Beads were collected by centrifugation, washed three times in NP-40 lysis buffer, and resuspended in SDS-sample buffer. Precipitated proteins were separated by SDS-PAGE and visualized by autoradiography.

30

6.2. RESULTS

6.2.1. ISOLATION OF NPI-1

The interactive trap was used to identify proteins which specifically interact with the

35

influenza A virus nucleoprotein (NP). The interactive trap is one of several genetic systems recently developed which uses the modular nature of

transcription activators to detect protein:protein interactions (Chien, et al., 1991, Proc. Natl. Acad.

5 Sci. USA 88: 9578-9582; Dalton & Treisman, 1992, Cell 68: 597-612; Durfee, et al., 1993, Genes Dev. 7: 555-569; Gyuris, et al., 1993, *supra*; Vojtek, et al., 1993, Cell 74: 205-214; Zervos, et al., 1993, *supra*).

The interactive trap consists of three components:

- 10 (1) a reporter gene that has no basal transcription;
(2) a fusion protein which contains a LexA DNA binding domain that is transcriptionally inert; and
(3) proteins encoded by an expression library, which are expressed as fusion proteins containing an
15 activation domain (Fig. 1A). Interaction of the LexA fusion protein and the fusion protein containing the activation domain will constitute a bimolecular transcriptional activator which, in this case, will confer the ability to grow on media lacking leucine
20 (Gyuris, et al., 1993, *supra*; Zervos, et al., 1993, *supra*). In the absence of this interaction the leu2 gene is not transcribed.

The NP gene of influenza A/PR/8/34 virus was subcloned as a translational fusion gene with the LexA
25 gene into pEG202 to generate pLexA-NP (Fig. 1B).

Strain R100 (Table II), which contains pLexA-NP, was transformed with a HeLa cell cDNA library constructed in pJG4-5. pJG4-5 contains an activation domain under control of a GAL1 promoter (Gyuris, et al., 1993,
30 *supra*).

TABLE II
YEAST STRAINS USED

35 Strains Genotype

EGY48	Mata trp1 ura3 his3 LEU2::pLEXAop6-LEU2
R100	EGY48, pLexA-NP(TRP1)
R101	EGY48, pLexA-NP, pSH18-34(HIS3)
R102	EGY48, pRFHM1(TRP1), pSH18-34

5

Library plasmids were rescued from 100 leu⁺ colonies. Reproducibility of the interaction of the NP with the
10 encoded library proteins was tested by transforming library plasmids into strain R101. Transformants were screened for galactose-dependent β -galactosidase activity and growth on media lacking leucine. Specificity for NP was analyzed by checking the
15 ability of library plasmids to confer growth on leu⁺ media and β -galactosidase activity in connection with a different LexA fusion plasmid, pRFHM1, encoding a fragment of the *Drosophila melanogaster* bicoid protein. Twenty-three library plasmids were confirmed
20 to encode NP-interactive proteins. Twelve identical 2.1 kbp clones encoded the carboxy terminal fragment of a protein termed nucleoprotein interactor 1 (NPI-1). Partial DNA sequencing showed that NPI-1 is the human homologue of the yeast SRP1 gene (*infra*).

25

6.2.2. CLONING AND SEQUENCING OF THE NPI-1 cDNA

The 2.1 kbp NPI-1 cDNA in pJG4-5 was sequenced by standard protocols. The 5' cDNA terminus of the NPI-1 gene was cloned by 5' RACE. cDNAs from 3
30 independently derived NPI-1 5'RACE products were cloned and sequenced. Nucleotide and derived amino acid sequences of NPI-1 are shown in Figure 2. The sequence reveals a 2.9 kbp cDNA which encodes a protein of 527 amino acids with a calculated molecular
35 weight of 58,754 Da and a pI = 4.74. The carboxyl

terminal 265 amino acids were encoded by the interactive trap library plasmid and interact with the viral NP.

Comparison of the deduced amino acid sequences in the GenBank and EMBL data bases using the FASTA and
5 TFASTA programs (Deveraux, et al., 1984, Nucleic Acids Res. 12: 387-395) demonstrated that NPI-1 is the human homologue of the *Saccharomyces cerevisiae* protein SRP1 (Yano, et al., 1992, Mol. and Cell. Biol. 12: 5640-5651). SRP1 was cloned as an allele-specific
10 suppressor of ts mutations in the zinc-binding domain of the A190 subunit of RNA polymerase I. The amino acid sequence is highly conserved between NPI-1 and SRP1: 50% identity and 81% similarity at the amino acid level. The amino terminus of NPI-1 has a
15 potential nuclear localization signal (Chelsky, et al., 1989, Mol. Cell. Biol. 9:2487-2492); amino acids 25 to 49 are rich in arginine, and contain a stretch of four consecutive arginines at amino acids 28 to 31. NPI-1, like SRP1, contains a series of 8
20 consecutive ARM motifs, which are 42 amino acid protein subsequences originally identified in the *Drosophila* armadillo protein (Peifer et al., Cell 76: 789-791, 1994; Yano, et al., 1992, *supra*) (Fig. 3, *infra*).

25

6.2.3. NPI-1 BINDS TO NP IN VITRO

In order to demonstrate that the NPI-1 binds to the viral NP, the NPI-1 cDNA fragment (amino acids 262 to 527) was subcloned into the bacterial expression
30 vector pGEX-5X-1 yielding a glutathione S-transferase fusion gene. The expressed fusion protein was purified from bacterial lysates on glutathione agarose beads. NP, which had been partially purified with the viral polymerase from influenza A/PR/8/34 virus was
35 specifically precipitated from solution by glutathione

agarose beads complexed with GST-NPI-1 (Fig. 4). The NP band migrates slightly faster than that of the GST-NPI-1 fusion protein. The identity of this protein was confirmed by immunoblot analysis using the anti-NP monoclonal antibody HT103 (Fig 4, lane 8).

5

6.2.4. IMMUNODETECTION OF NPI-1 IN CELL EXTRACTS

Rabbit antisera raised against GST-NPI-1 were used to identify a polypeptide from total cellular extracts of both HeLa and MDBK cells with an apparent
10 molecular weight of 65 kDa (Fig. 5). The molecular weight predicted from the derived amino acid sequence of the cDNA is slightly smaller (59 kDa). A lower amount of NPI-1 was present in the cytoplasmic
15 fraction generated by lysis of cells in the presence of NP-40 than in the total cellular extract suggesting that most of NPI-1 is located in the nucleus (Fig. 5). This is consistent with results localizing the NPI-1 homologue SRP1 to the nucleus of yeast cells by immunofluorescence (Yano, et al., 1992, *supra*).
20 Localization of NPI-1 to a particular intracellular compartment by immunofluorescence experiments has not been possible due to the high background fluorescence of the antisera preparations used.

25 6.2.5. NPI-1 INTERACTS WITH NP IN INFECTED CELLS

Since NP formed a complex with NPI-1 *in vitro*, we examined whether NP and NPI-1 form a complex in infected cells. NP was specifically
coimmunoprecipitated from extracts of influenza A/WSN
30 virus infected HeLa cells by antisera directed against NPI-1 (Fig 6). This demonstrates an interaction of the viral NP and the cellular NPI-1 during influenza A virus infection.

35

7. Example: THE IDENTIFICATION OF NS1I-1 AND ITS
INTERACTION WITH INFLUENZA
NUCLEOPROTEIN NS1

In the example described below, the yeast interactive trap system was used to identify a human protein, NS1I-1 (NS1-interactor-1), from a HeLa cell
5 cDNA library on the basis of its binding to NS1 of influenza A virus. NS1I-1 is shown herein to be recognized not only by NS1 proteins from five human and avian influenza A strains, but also by NS1 of
10 influenza B virus. Surprisingly, NS1I-1 is homologous to a steroid dehydrogenase isolated from pigs (Leenders, et al., 1994, Eur. J. Biochem. 222: 221-227). Several proteins with a dehydrogenase function have recently been shown not only to have enzymatic
15 activity but also to be involved in post-transcriptional events of gene-expression (Hentze, 1994, supra). This strong conservation supports an important functional role of the NS1I-1 interaction during the viral life cycle.

20

7.1. MATERIALS AND

METHODS

7.1.1. YEAST, E.COLI STRAINS, AND PLASMIDS

25 Manipulations of nucleic acids, *Escherichia coli* and yeast followed essentially standard procedures as described elsewhere (Ausubel, et al., 1992, Current Protocols in Molecular Biology, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York). The yeast strains EGY40 (Mata *trp1*
30 *ura3 his3*) and EGY48 (Mata *trp1 ura3 his3 LEU2::pLEX-Aop6-LEU2*) as well as plasmids pEG202, pRFHM1, and pSH18-34, and the HeLa cell cDNA constructed in pJG4-5 have been described (Gyuris, et al., 1993, supra; Zervos, et al., 1993, supra). *E. coli* strains used
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for cloning and expression were MH3 (trpC araD lacX hsdR galU galK), DH5 α (F ϕ 80dlacZ Δ M15 Δ (lacZY-argF)U169 deoR recA1 endA1 hsdR17(r_k-m_k+) supE44 λ -thi- gyrA96 relA1), and BL26 (F_{ompT} hsdS_B(r_B-m_B-) gal dcm). pLexA-NS1 was constructed by subcloning the cDNA of the NS segment of influenza virus A/PR/8/34 downstream of the LexA gene in pEG202. pGEX-NS1I-1 was constructed by subcloning the HeLa cDNA-insert of library plasmid pK5 as an EcoRI/XbaI-fragment into pGEX-5X-1 (Pharmacia). DNA-oligonucleotides used were: GSP-I, 5'-
10 dTCCTGATGTTGCTGTAGACG-3',
GSP-II, 5'-dGCACGACTAGTATGATTTGC-3', and the 5'RACE anchor primer (BRL), 5'-
dCUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'

15 7.1.2. IDENTIFICATION OF NS1-INTERACTORS

The interactive trap selection was performed essentially as described for NPI-1 in Section 6.1.2, above. The selection strain was constructed by transforming EGY48 with the bait plasmid pLexA-NS1 and
20 the lacZ-reporter plasmid pSH18-34. Expression of lacZ from pSH18-34 is transcriptionally controlled by a GAL1 promoter and LexA-dependent operator sites. A HeLa cell cDNA library was introduced into the selection strain using the lithium acetate method
25 (Ito, et al., 1983, *supra*). Primary transformants were selected on trp^{his}ura⁻ glucose plates. 1 x 10⁶ cells representing 3.3 x 10⁵ independent transformants were plated on 150 mm trp^{his}ura⁻leu⁻-galactose plates to select for clones expressing NS1-interacting
30 proteins. Viable cells were replica-transferred to a nitrocellulose filter and assayed for β -galactosidase activity using 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) as described (Ausubel et al., 1992, *supra*). Positive clones were tested in a second
35

round of selection by replica plating onto X-gal trp⁺ his⁺ura⁺ galactose plates. Plasmid DNA was isolated from yeast clones expressing β -galactosidase activity only on galactose plates and library plasmids were recovered by transformation into *E.coli* MH3 as
5 described in Section 6.1.2, above. The specificity of the isolated plasmids was tested by co-transformation with pLexA-NS1 or pRFHM1 into EGY40 harboring pSH18-34. pRFHM1 expresses an unrelated LexA-bicoid fusion protein. The resulting strains were assayed for β -
10 galactosidase activity on X-gal trp⁺his⁺ura⁺ plates containing glucose or galactose. Plasmids that induced β -galactosidase only in the presence of galactose and only in conjunction with pLexA-NS1 were considered to encode true interacting proteins.

15

7.1.3. CLONING OF NS1I-1 5'-END CDNA

Cloning of cDNA derived from the 5'-end of NS1I-1 mRNA followed a RACE-procedure (rapid amplification of cDNAs ends) (Frohmanm, et al., 1988,
20 *supra*) using a 5'RACE-kit (BRL). First strand cDNA was synthesized from 1 μ g of HeLa cell poly(A)-RNA hybridized to 2.5 pmol NS1I-1-specific oligonucleotide GSP-I using reverse transcriptase. The cDNA was
25 tailed at the 5'-end with dC by terminal transferase. The product was used as a template for the amplification of a 5'RACE-product by PCR using a nested oligonucleotide GSP-II and an anchor primer provided by the kit. The resulting fragment was
30 subcloned in pGEM-T (Promega) to form pRACENS1I-1, and sequenced by the standard dideoxy method. The NCBI-search was conducted using Fasta, Tfasta. Sequence comparison was conducted using Bestfit.

35

7.1.4. NORTHERN BLOT ANALYSIS

1 µg of HeLa cell poly(A)-RNA was separated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran, Amersham), and UV-crosslinked. The RNA was hybridized to a ³²P-labeled, NS1I-1-specific probe derived from a fragment (corresponding to positions +791 to +1745) of the original pK5 library isolate as described (Ausubel, et al., 1992, supra).

7.1.5. VIRUSES, CELLS, AND EXTRACTS

Influenza strains A/WSN/33 (H1N1), A/Berkeley/1/68 (H2N2), A/Beijing/32/92 (H3N2), A/duck/Alberta/76 (N12N5), A/turkey/Oregon/71 (H7N5), and B/Lee/40 were grown in the allantoic cavity of 10 days old embryonated chicken eggs. Confluent monolayers of Madin Darby canine kidney-(MDCK)-cells were infected with influenza viruses at an m.o.i. of 10 for one hour in 35 mm dishes. Infection was continued at 37°C (influenza A viruses) or 35°C (influenza B/Lee/40) for 5 hours in MEM-medium containing 0.1% bovine serum albumin. Cells were labeled with 100 µCi of ³⁵S-methionine and ³⁵S-cysteine (ICN) per dish for one hour in MEM-met-cys-medium. Cells were washed and scraped in ice-cold phosphate buffered saline (PBS). Cells from one dish were lysed with 500 µl NET-N buffer (10mM Tris/HCL pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.05% Nonidet P 40) and two 30 second pulses in a Raytheon sonicator at a setting of 1A. Lysates were centrifuged for 10 minutes at 20,000 rpm in a TL100.3 rotor. The supernatants were used for precipitation of proteins.

7.1.6. EXPRESSION OF GST-NS1I-1 FUSION PROTEIN IN E. COLI AND PRECIPITATION OF VIRAL PROTEINS FROM CELL EXTRACTS

NS1I-1 was expressed in *E. coli* BL26 from pGEX-NS1I-1 as a GST (glutathione-S-transferase)-NS1I-

1 fusion protein with a predicted molecular weight of
77 kDa. Production of GST-NS1I-1 was induced using
isopropyl- β -D-galactopyranoside essentially as
described (Smith, et al., 1988, *supra*). GST-NS1I-1
was adsorbed from bacterial lysates to glutathione
5 sepharose beads (Pharmacia) as recommended by the
manufacturer. Beads were washed three times with PBS
to remove contaminating proteins. 10 μ l of
glutathione sepharose coated with GST-NS1I-1 fusion
protein was rotated with 100 μ l extract of virus-
10 infected MDCK-cells (see above) in 750 μ l NET-100
buffer (20 mM Hepes, pH 8.0, 100mM NaCl, 0.5 mM DTT)
for 90 minutes at 4°C. The beads were washed three
times with PBS/0.05% NP-40 and precipitated proteins
were analyzed by SDS-gel electrophoresis and
15 autoradiography. In parallel reactions, viral
proteins were immunoprecipitated from 50 μ l of
infected cell extracts using 5 μ l of anti-NS1 or anti-
M1 antiserum and protein A agarose as described
(Harlow & Lane, 1988, *supra*). As a negative control,
20 GST protein was expressed in BL26 from pGEX-5X-1 and
used the same way in the co-precipitation assay.

7.2. RESULTS

25 7.2.1. ISOLATION OF NS1-INTERACTING FACTORS

The yeast interaction trap system (Gyuris,
et al., 1993, *supra*; Zervos, et al., 1993, *supra*) was
used to identify cellular proteins that interact with
the non-structural protein NS1 of influenza A virus.
30 A LexA-NS1 fusion protein was used as bait to screen
library in which HeLa cell cDNAs were expressed as
fusions with an acidic transcription activation domain
(Gyuris, 1993 #159). Colonies were selected, in which
either of two reporter genes, LEU2 and lacZ, were
35 activated by the cDNA-encoded proteins. This double

selection scheme was used to increase the stringency, because in an initial screen a high proportion of candidates scored negative in subsequent genetic tests. The library plasmids were isolated from the selected clones.

5 The binding specificity of the encoded fusion proteins was tested by assaying the activation of a lacZ-reporter gene encoded on pSH18-34. Expression of β -galactosidase from this plasmid is transcriptionally controlled by LexA-specific operator
10 sites. The isolated library plasmids were co-transformed with pLexA-NS1 or pRFHM1 into EGY40 harboring pSH18-34. pRFHM1 expresses a LexA-bicoid fusion protein and was used as a non-specific operator-binding control. The resulting strains were
15 assayed for β -galactosidase activity specifically on X-gal plates containing galactose, but not glucose. From 3.3×10^5 independent library transformants, three plasmids were isolated that induced galactose-specific activation of the lacZ reporter gene only in
20 combination with pLexA-NS1. Sequence analysis indicated that the three plasmids were each derived from different cellular cDNAs.

7.2.2. CLONING AND SEQUENCE ANALYSIS OF NS1I-1

25 One of the isolated plasmids, pK5, was analyzed further. It carried a cDNA-insert of 1781 bp with an open reading frame of 1413 nucleotides followed by 368 nucleotides of a potentially untranslated region (Fig. 12). The cDNA terminated
30 with an oligo(A)-tract and had a consensus poly(A)-site at positions 2526-2531. Northern blot analysis of HeLa cell poly(A)-RNA using a NS1I-1-specific probe detected one single transcript of about 3.0 kb suggesting that the pK5 insert represented an
35 incomplete cDNA (Fig. 13). The remaining NS1I-1 cDNA

was cloned by a 5'RACE procedure (Frohman, et al., 1988, *supra*). Four independent clones were sequenced that differed only in length at the very 5'-end. The longest 5'RACE product, contained in pRACENS1I-1, extended the NS1I-1 sequence for 893 nucleotides
5 upstream totalling in a cDNA of 2674 bp (Fig. 12). The sequence has one long open reading frame encoding a protein of 735 amino acids with a predicted molecular mass of 79.5 kDa and a pI of 9.06. The putative ATG-start codon is located 103 nucleotides
10 downstream of the 5'-end and is in the context of a sequence consistent with its being a translational start (Kozak, 1989, J. Cell Biol. 108: 229-241).

Sequence comparisons through the EMBL- and Genbank databases using the FASTA- and TFASTA-analysis
15 programs revealed that NS1I-1 is highly homologous to porcine 17 β -estradiol dehydrogenase (Leenders, et al., 1994, *supra*). The two cDNAs are 86% identical on the nucleic acid level. The encoded proteins are 84% identical and are 92% similar when allowing for
20 conserved amino acid substitutions. NS1I-1 cDNA also shows strong homology to ten human cDNA fragments that have been isolated as expressed sequence tags, as revealed by a BLAST-analysis of the NCBI-database (fragments are between 134 to 556 bp in length).
25 These cDNAs were derived from different tissues including liver, spleen, brain, adipose tissue, and adrenals tissue indicating a broad expression of NS1I-1 in the body.

The encoded NS1I-1 protein features two
30 conserved sequence motifs of the short-chain alcohol dehydrogenase family (Persson, et al., 1991, Eur. J. Biochem. 200: 537-543). Specifically, amino acids 15 to 22 (TGAGAGCG) are similar to the potential co-factor binding site, and residues 163 to 167 (YSAAK)
35 correspond to a short stretch that has been suggested

to participate in catalysis (Chen, et al., 1993, Biochemistry 32: 3342-3346). The presence of the tri-peptide AKL at the carboxy-terminus was also noted. Similar tri-peptide motifs have been found to serve as targeting signals for import into microbodies (for a
5 review, see de Hoop & Ab, 1992, Biochem. J. 286: 657-669). However, the presence of this signal does not automatically direct a protein to these organelles (de Hoop & Ab, 1992, *supra*).

10 7.2.3. NS1I-1 BINDS NS1 PROTEIN FROM EXTRACTS OF INFLUENZA VIRUS INFECTED CELLS

In order to confirm a physical interaction between NS1I-1 protein and NS1 expressed in influenza virus infected cells, a co-precipitation assay was
15 performed as similarly described in Section 6.2.3, above, for NPI-1. A glutathione-S-transferase(GST)-NS1I-1 fusion gene was constructed and expressed in *E.coli*. GST-NS1I-1 fusion protein from bacterial lysate was absorbed to the affinity matrix glutathione
20 agarose and purified from contaminating bacterial proteins. The immobilized fusion protein was used to bind and precipitate ³⁵S-labeled proteins from extracts of MDCK cells infected with human influenza A/WSN/33 viruses (Fig. 14). The NS1 protein of this strain is
25 98% identical to its counterpart from A/PR/8/34 used in the yeast interaction screen. Aliquots of the same extract were used to in parallel reactions to immunoprecipitate influenza virus proteins NS1 and M1. The precipitated proteins were analyzed by SDS-gel
30 electrophoresis and visualized by fluorography. Fig. 14 shows, that GST-NS1I-1 efficiently precipitated a protein band co-migrating with immunoprecipitated NS1 protein from infected cell extract (compare lanes 2 and 3). This interaction was specific for NS1I-1
35 since no proteins were detected in precipitates using

GST only (lane 6). In addition, no proteins were precipitated by GST-NS1I-1 from mock-infected cells (lane 8), showing that a virus induced protein was recognized by NS1I-1. This experiment confirmed, that NS1I-1 interacts specifically with the NS1 protein of influenza A virus.

If this interaction is important for the viral life-cycle one would expect it to be conserved. Consequently, the binding of NS1I-1 to NS1 proteins from other influenza A strains should be detectable despite of their considerable variation in the primary structure (Baez, et al., 1981, Virology 113: 397-402; Ludwig, et al., 1991, Virology 183: 566-577). Therefore the interaction between NS1I-1 and NS1 was examined using the same co-precipitation assay described above, with extracts from cells infected with different influenza A and B virus strains.

Mutations accumulate in the NS1 gene at a steady rate over time (Buonagurio, et al., 1985, Science 232: 980-982). Thus, the time-span between the isolation of two strains is reflected in the sequence variation of its NS1 proteins (Ludwig, et al., 1991, *supra*; Buonagurio, et al., 1985, *supra*). NS1I-1 binding to NS1 proteins from two recently isolated human influenza A strains A/Beijing/32/92 and A/Berkeley/1/68 was examined. As can be seen in Fig. 15, Panels C and D, respectively, NS1 proteins from both strains were specifically precipitated (Fig 15, Panels C and D, lanes "GST-K5"). A low immunoprecipitation efficiency of NS1 protein from the Beijing-strain (Panel C) was reproducibly observed. The NS1 proteins of A/Berkeley/1/68 and A/WSN/33 are 90.8% identical to each other. The NS1 sequence of A/Beijing/32/92 is not known.

The following analyses were conducted to examine whether GST-NS1I-1 is also recognized by the

more divergent NS1 proteins of the avian influenza strains A/duck/Alberta/76 and A/turkey/Oregon/71. The NS1 proteins of these strains are 66.5% and 63.6% identical, respectively, to A/WSN/33. Significantly, NS1 of A/turkey/Oregon/71 is only 124 amino acids in length, lacking most of the carboxy-terminal half of other NS1 proteins, which consist of 207 to 237 amino acids (Norton, et al., 1987, Virology 156: 204-213). Nevertheless, precipitation of a protein band co-migrating with NS1 from both strains was observed (Fig. 15, Panels A and B, lanes "GST-K5"). The NS1 and M1 proteins of A/duck/Alberta/76 could not be separated by the gel system used. Significant amounts of nucleoprotein in the GST-NS1I-1 precipitates of these avian strains were reproducibly detected for undetermined reasons.

Finally, the co-precipitation assay was used to test the human influenza B virus B/Lee/40. Surprisingly, GST-NS1I-1 precipitated specifically the influenza B virus NS1 protein, although it is only 20.6% identical to NS1 from A/WSN/33 (Fig. 15, Panel E, lane "GST-K5"). Taken together, the binding of GST-NS1I-1 to NS1 proteins expressed by several influenza A and B virus strains could be demonstrated, despite the great divergence of their primary structures. This result strongly supports an important function of this interaction during the viral life cycle, and indicates that the NS1I-1 interaction is an excellent target for antiviral intervention.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in

addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page __, lines __ of the description '

A. IDENTIFICATION OF DEPOSIT '

Further deposits are identified on an additional sheet '

Name of depositary institution '

American Type Culture Collection

Address of depositary institution (including postal code and country) '

**12301 Parkdawn Drive
Rockville, MD 20852
US**Date of deposit ' November 30, 1994 Accession Number ' 75950**B. ADDITIONAL INDICATIONS '** (leave blank if not applicable). This information is continued on a separate attached sheet**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE '** (if the indications are not all designated States)**D. SEPARATE FURNISHING OF INDICATIONS '** (leave blank if not applicable)

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Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

Accession No.

75951

Date of Deposit

November 30, 1994

WHAT IS CLAIMED IS:

1. An assay for identifying a substance that inhibits the specific interaction of a host cell protein, that is not a cell surface receptor protein, with a viral protein required for viral infection,
5 replication, assembly or release, comprising:

(a) contacting a protein or peptide containing an amino acid sequence corresponding to the binding site of the host cell protein with a protein or peptide having an amino acid sequence corresponding
10 to the binding site of the viral protein, under conditions and for a time sufficient to permit binding and the formation of a complex, in the presence of a test substance, and

(b) detecting the formation of a complex,
15 in which the ability of the test substance to inhibit the interaction between the host cell protein and the viral protein is indicated by a decrease in complex formation as compared to the amount of complex formed in the absence of the test substance.

20

2. An assay for identifying a substance that inhibits the interaction of influenza virus NP with a host cell protein comprising:

(a) contacting a protein or peptide
25 containing an amino acid sequence corresponding to the binding site of influenza virus NP with a protein or peptide containing an amino acid sequence corresponding to the binding site of the host cell protein, under conditions and for a time sufficient to
30 permit binding and formation of a complex, in the presence of a test substance, and

(b) detecting the formation of a complex, in which the ability of a test substance to inhibit the interaction between influenza virus NP and the host
35 cell protein is indicated by a decrease in complex

formation as compared to the amount of complex formed in the absence of the test substance.

3. The assay of Claim 2 in which the host cell protein is NPI-1.

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4. The assay of Claim 3 in which the host cell protein is NPI-2.

5. The assay of Claim 3 in which the host cell protein is NPI-3.

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6. The assay of Claim 3 in which the host cell protein is NPI-4.

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7. The assay of Claim 3 in which the host cell protein is NPI-5.

8. The assay of Claim 3 in which the host cell protein is NPI-6.

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9. An assay for identifying a substance that inhibits the interaction of influenza virus NS1 with a host cell protein comprising:

(a) contacting a protein or peptide containing an amino acid sequence corresponding to the binding site of influenza virus NS1 with a protein or peptide containing an amino acid sequence corresponding to the binding site of the host cell protein, under conditions and for a time sufficient to permit binding and formation of a complex, in the presence of a test substance, and

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(b) detecting the formation of a complex, in which the ability of a test substance to inhibit the interaction between influenza virus NS1 and the host cell protein is indicated by a decrease in complex

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formation as compared to the amount of complex formed in the absence of the test substance.

10. The assay of Claim 9 in which the host cell protein is NS1I-1.

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11. The assay of Claim 1, 2, or 9 in which one protein or peptide of the complex is immobilized, and the other protein or peptide is labeled with a signal-generating compound.

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12. The assay of Claim 11 in which an immobilized antibody is used to anchor the immobilized protein or peptide.

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13. The assay of Claim 11 in which a labeled antibody is used to label the protein or peptide with a signal-generating compound.

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14. The assay of Claim 11 in which the protein or peptide substrate is immobilized prior to the reaction so that the reaction is conducted in a solid-liquid phase.

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15. The assay of Claim 1, 2, or 9 in which the proteins or peptides are contacted in a liquid phase to form a complex which is separated from the liquid phase at the end of the reaction.

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16. The assay of Claim 15, in which the complex formed is separated from the liquid phase by immobilizing the complex on a solid phase.

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17. The assay of Claim 16 in which the complex is captured by an immobilized antibody specific for one of the proteins or peptide binding partners.

18. A method for treating influenza virus infection, comprising administering to an infected individual, a therapeutically effective amount of a substance that inhibits the specific interaction of a host cell protein with a viral protein required for
5 viral infection, replication, assembly or release.

19. The method of Claim 18 in which the viral protein is NP.

10 20. The method of Claim 18 the host cell protein is NPI-1.

21. The method of Claim 18 the host cell protein is NPI-2.
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22. The method of Claim 18 the host cell protein is NPI-3.

23. The method of Claim 18 the host cell protein is NPI-4.
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24. The method of Claim 18 the host cell protein is NPI-5.

25 25. The method of Claim 18 the host cell protein is NPI-6.

26. The method of Claim 18 the viral protein is NS1.
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27. The method of Claim 18 the host cell protein is NS1I-1.

28. An isolated DNA sequence which encodes the amino acid sequence of NPI-1, or which selectively
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hybridizes to the complement of the coding sequence of NPI-1 and encodes a functionally equivalent gene product.

29. An isolated DNA sequence which encodes the
5 complement of the DNA sequence of Claim 28.

30. A DNA vector containing the DNA sequence of Claim 28.

10 31. A DNA vector containing the DNA sequence of Claim 29.

32. An expression vector containing the DNA
sequence of Claim 28 operatively associated with a
15 regulatory element that directs the expression of the DNA sequence.

33. A genetically engineered host cell
containing the DNA sequence of Claim 28 operatively
20 associated with a regulatory element that directs expression of the DNA sequence in the host cell.

34. A DNA sequence which encodes the amino acid
sequence of NPI-2, NPI-3, NPI-4, NPI-5, or NPI-6 or
25 which selectively hybridizes to the complement of the coding sequence of NPI-2, NPI-3, NPI-4, NPI-5, or NPI-6 and encodes a functionally equivalent gene product.

35. A DNA sequence which encodes the complement
30 of the DNA sequence of Claim 34.

36. A DNA vector containing the DNA sequence of Claim 34.

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37. A DNA vector containing the DNA sequence of Claim 35.

38. An expression vector containing the DNA sequence of Claim 34 operatively associated with a regulatory element that directs the expression of the DNA sequence.

39. A genetically engineered host cell containing the DNA sequence of Claim 34 operatively associated with a regulatory element that directs expression of the DNA sequence in the host cell.

40. An isolated DNA sequence which encodes the amino acid sequence of NS1I-1, or which selectively hybridizes to the complement of the coding sequence of NS1I-1 and encodes a functionally equivalent gene product.

41. An isolated DNA sequence which encodes the complement of the DNA sequence of Claim 40.

42. A DNA vector containing the DNA sequence of Claim 40.

43. A DNA vector containing the DNA sequence of Claim 41.

44. An expression vector containing the DNA sequence of Claim 40 operatively associated with a regulatory element that directs the expression of the DNA sequence.

45. A genetically engineered host cell containing the DNA sequence of Claim 40 operatively

associated with a regulatory element that directs
expression of the DNA sequence in the host cell.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06347

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/70; C12N 5/00, 15/00; A01N 37/18; C07H 21/04

US CL : 435/5,240,2,320.1; 514/2; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5,240,2,320.1; 514/2; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W. K. Joklik et al, "Zinsser Microbiology," published 1992 by Appleton and Lange (Norwalk, Connecticut), pages 854-861, see entire document.	1,2,11-17
A	Virus Research, Vol. 1, No. 6, issued September 1984, S. Nakada et al., "Complete nucleotide sequence of the influenza C/California/78 virus nucleoprotein gene," pages 433-441, see entire document.	1-8, 11-17, 38-39



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 AUGUST 1995

Date of mailing of the international search report

29 AUG 1995

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06347

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	The EMBO Journal, Vol. 13, No. 13, issued 1994, F. Baudin et al., "Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent," pages 3158-3165, see entire document.	1-8, 11-17, 28-39

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/06347

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-8, 11-17, 28-39

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-8, 11-17, 28-39, drawn to a first product and assay.

Group II, claims 9-17, 40-45, drawn to a second product and assay.

Group III, claims 18-25, drawn to treatment method involving first product.

Group IV, claims 26, 27, drawn to treatment method involving second product.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I and II lack the same or corresponding special technical feature because the claims of Group II are drawn to a distinctly different product from the product of Group I. Groups I and III lack the same or corresponding technical features because the method of Group III is a distinctly different method from the method of Group I, requiring different method steps and reagents. Groups II and IV lack the same or corresponding special technical feature because the method of Group IV is a distinctly different method from the method of Group II requiring different method steps and reagents. PCT Rule 13.2 does not provide for multiple products or methods.